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Impact of host and environmental factors on β-glucuronidase enzymatic activity: implications for gastrointestinal serotonin

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Abstract

The gastrointestinal tract houses a reservoir of bacterial-derived enzymes which can directly catalyze the metabolism of drugs, dietary elements and endogenous molecules. Both host and environmental factors may influence this enzymatic activity, with the potential to dictate the availability of the biologically-active form of endogenous molecules in the gut and influence inter-individual variation in drug metabolism. We aimed to investigate the influence of the microbiota, and the modulation of its composition, on fecal enzymatic activity. Intrinsic factors related to the host, including age, sex and genetic background, were also explored. Fecalase, a cell-free extract of feces, was prepared and used in a colorimetric-based assay to quantify enzymatic activity. To demonstrate the functional effects of fecal enzymatic activity, we examined β-glucuronidase-mediated cleavage of serotonin β-D-glucuronide (5-HT-GLU) and the resultant production of free 5-HT by HPLC. As expected, β-glucuronidase and β-glucosidase activity were absent in germ-free mice. Enzymatic activity was significantly influenced by mouse strain and animal species. Sex and age significantly altered metabolic activity with implications for free 5-HT. β-glucuronidase and β-glucosidase activity remained at reduced levels for nearly two weeks after cessation of antibiotic administration. This effect on fecalase corresponded to significantly lower 5-HT levels as compared to incubation with pre-antibiotic fecalase from the same mice. Dietary targeting of the microbiota using prebiotics did not alter β-glucuronidase or β-glucosidase activity. Our data demonstrate that multiple factors influence the activity of bacterial-derived enzymes which may have potential clinical implications for drug metabolism and the deconjugation of host-produced glucuronides in the gut.

New and Noteworthy

This paper explores a comprehensive range of host and environmental related factors which introduce variability in the expression of bacterial-derived metabolic enzymes. Our results demonstrate that altered β-glucuronidase activity has implications for the bioavailability of luminal serotonin. The experimental approach employed, fecalase, provides a mechanistic basis and translational platform to further delineate the functional outputs of altered metabolic activity, and the associated physiological effects of microbiota-targeted interventions on host response to drugs and host-produced glucuronides.

Keywords: *microbiome, enzyme activity, glucuronidation, serotonin, β-glucuronidase*

Introduction

In the quest to usher in a new era of precision medicine, a more comprehensive understanding of the intricate factors driving inter-individual variation in drug metabolism is an essential stepping stone. As the drug development process has traditionally focused solely on the liver as the primary site of drug metabolism, the modulation of host drug-metabolizing enzymes has been considered one key source of variability in drug pharmacokinetics (44). The gastrointestinal (GI) tract, however, houses a reservoir of bacterial-derived enzymes, including β-glucuronidase and β-glucosidase, that can also directly catalyze the metabolism of foreign substances ("xenobiotics") (6, 63), as well as endogenous compounds ("endobiotics") (45). Bacterial-mediated cleavage of host-derived glucuronides can thus act to regulate levels of endobiotics, including serotonin (5-HT) (23, 39); 5-HT functions within the gut as a paracrine factor, a pro-inflammatory signaling molecule, endocrine hormone, neurotransmitter and growth factor (18). Moreover, β-glucuronidase and β-glucosidase are both members of the glycosidase family of enzymes that are also involved in the breakdown of complex carbohydrates which are a source of carbon for bacterial growth (11). The metabolic activity of the gut microbiome has been conceptually equated with being greater than that of the liver (8) and may be equally important for the metabolism of some drugs such as lovastatin (68). Host, and environmental factors, which alter the expression of these bacterial-derived metabolic enzymes, may contribute to inter-individual variation in the metabolism of both xenobiotics and endobiotics, with potential effects on therapeutic outcomes and host physiological processes, respectively.

Gut-derived microbial enzymes are linked to a broad range of functions, including roles in the metabolism of amino acids, fatty acids, and carbohydrates. The additional role of microbial enzymes in xenobiotic metabolism is gaining traction (8, 33). The most commonly examined

bacterial enzymes are those involved in the production of toxic, mutagenic, or carcinogenic metabolites, namely β-glucuronidase, β-glucosidase, nitroreductase, and azoreductase (43). β-glucuronidase and β-glucosidase are the most predominant and exhibit the most influential activity across species (49). β-glucosidase and β-glucuronidase catalyze the hydrolysis of glycosidic bonds either, to terminal non-reducing residues in β-D-glucosides and oligosaccharides, or the cleavage of a glycosidic bond between glucuronic acid and either small molecules or polysaccharides (45, 55). The conservation of microbial β-glucuronidase across the major GI bacterial phyla, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, highlights the crucial role this enzyme exerts on chemical dynamics in the gut (45).

β-glucuronidase is amongst the most studied bacterial drug-metabolizing enzymes due to its role in the deconjugation of hepatically-glucuronidated metabolites. Glucuronidation of endo- or xeno-biotics occurs primarily in the liver, and following biliary secretion to the intestine, glucuronides are subject to excretion, or metabolism by bacterial-derived β-glucuronidase enzymes (46). The latter effect reactivates the aglycone form of the compound, which can be excreted, or undergo enterohepatic recirculation (45) with either beneficial or deleterious effects on the host (12, 19). Host and environmental factors, which alter the expression of this enzyme, may, therefore, aid the understanding of fundamental metabolic processes.

Fecal samples, more specifically, the enzyme fraction of feces referred to as fecalase (56), reflect the gut microbial enzymatic activity (21). Previous data has shown inter-individual variation in enzymatic activity, at least in part, due to differences in the composition of the gut microbiota or as a consequence of other influencing factors such as diet and antibiotic treatment (41).

In this study, we investigated enzymatic activity in feces collected from several species and investigated the impact of sex, age, and genetic background on β-glucuronidase and β-

glucosidase activity. To examine the influence of the microbiota on fecal enzymatic activity, we used germ-free (GF) animals and studied the impact of antibiotics and a prebiotic mix on the metabolic activity of the gut microbiota.

Materials and Methods

Ethical approval and animal fecal sample collection

All experiments were conducted in accordance with the European Directive 86/609/European Economic Community and the Recommendation 2007/526/65/EC. Ethical approval (FOS-inulin and antibiotic study under B100/3774 approval) was obtained from the Animal Experimentation Ethics Committee of University College Cork before the commencement of all animal-related experiments.

Animals were housed in standard wire cages at 20-22ºC and 50 ± 10% humidity under a strict 12:12-h light/dark cycle in a conventional or GF animal facility. Conventional animals received standard laboratory chow and water *ad libitum* (see *FOS-inulin Study* for specific diet information). GF mice were housed in specific isolators and fed the autoclaved RM1-AP (Special Diets Services; Cas no. 801010). Following at least 1 wk habituation to the facility, feces were collected from C57BL/6, NIH Swiss Webster (10-12 wk), BALB/c (10-12 wk) and Sprague Dawley rats (8-10 wk) (Harlan or Envigo, UK). Additionally, fecal samples were collected from male caspase-1 knockout (KO), caspase wild-type (WT) and interleukin (IL)-10-deficient mice bred in-house on a C57BL/6 background (all ~ 10-12 wk). Each fecal sample was collected freshly within a defined period (9-11 am) and processed immediately after collection or stored at -80 ºC.

Landrace pigs were sourced locally and housed individually at the University's Biological Services Unit. Fecal matter was collected directly from the pig (10-12 wk) anorectal region at dissection and stored at -80 ºC until further processing.

Ethical approval and human fecal sample collection

Human fecal samples were collected from healthy controls (both male and female subjects; age range: 43-76 yr) as part of an ongoing APC Microbiome Ireland study (APC070) in conjunction with the Centre for Gerontology and Rehabilitation and was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Antibiotic-induced microbial disruption

Following 1-wk habituation, adolescent (i.e., 27 days old at baseline) and adult (i.e., 72 days old at baseline) C57BL/6 male mice were allocated into two wide-spectrum antibiotic-cocktail treatment groups and a control group (n=8 or n=9-12, respectively). Mice were randomly assigned to different cohorts using a random number generator. Mice in the same cage underwent the same treatment to avoid confounding factors such as coprophagic effect. These antibiotic cocktails have limited oral bioavailability and were previously described to ablate the gut microbiota (24, 30). The cocktails were administered in drinking water to avoid any adverse effects from chronic stress induced by alternative administration methods such as oral gavage. All antibiotics were purchased from Discovery Fine Chemicals Ltd and freshly prepared every second day with autoclaved water. The first antibiotic cocktail consisted of ampicillin (1mg/ml), vancomycin (5mg/ml) and neomycin (10mg/ml; CAS no. 1405-110-3) (herein referred to as ABX-3). The second antibiotic cocktail consisted of ampicillin (1mg/ml; CAS no. 69-52-3), vancomycin (5mg/ml; CAS no. 1404-93-9), ciprofloxacin hydrochloride (0.2mg/ml; CAS no. 93107-08-5), imipenem (0.25mg/ml; CAS no. 74431-23-5) and metronidazole (1mg/ml; CAS no. 443-48-1) (herein referred to as ABX-5). Mice were treated for 21 days. Controls received autoclaved water with no antibiotic. Adolescent mice were culled (by decapitation) at the cessation of antibiotic treatment, whereas adult mice were maintained for approximately 6 wk after the end of antibiotic-treatment to examine the recovery of the microbiota.

Prebiotic intervention study

Previous work by our laboratory explored whether prebiotic supplementation (fructo-oligosaccharide (FOS)-inulin) in middle-aged mice could reverse the signs of ageing on peripheral immune response and neuroinflammation (4). Supplementation with this 10% FOS-inulin dietary intervention has been shown to increase species richness, an important index of diversity, and significantly altered the composition of the gut microbiota and, as expected with prebiotics, promoted the growth of beneficial bacteria. The prebiotic mix increased the abundance of *Bifidobacterium*, *Akkermansia*, *Prevotellaceae UCG-001* and *Bacteroides* in middle-aged mice. On the other hand, FOS-inulin decreased the abundance of *Lactobacillus* and *Roseburia* (4). Here, we sought to examine the effects of prebiotic supplementation on the metabolic activity of the gut microbiota using fecalase prepared from the same animals. Conventional male C57BL/6 middle-aged (i.e., 10 mo) mice received standard diet (ssniff-Spezialdiäten; Cas no. S9912-S010) or diet supplemented with 10% FOS-enriched inulin (a mixture of 92±2% Inulin and 8±2% FOS; Orafti®Synergy1; Cas no. SYN1 A3-40*01-13). Mice were equally assigned to experimental groups based on bw to ensure equal distribution among the groups. Fecal pellets were collected after 5 wk of treatment, immediately snap frozen on dry ice and stored at -80 ^{o}C until further processing (n=9-10).

Fecalase preparation

Fecalase was prepared from freshly excreted or fresh-frozen feces according to a modified version of a previously published method (36). Briefly, the weighed fecal pellet was suspended in potassium phosphate buffer (0.01M, pH7.4) and homogenized using a mini Bead-Beater machine for 1.5 min (*Figure 1*). The fecal suspension was centrifuged at 2,000 rpm for 5 min. Centrifugation of the isolated supernatant was conducted at 10,000 rpm for 20 min, and the resulting supernatant (fecalase) was then used for the enzyme activity assay.

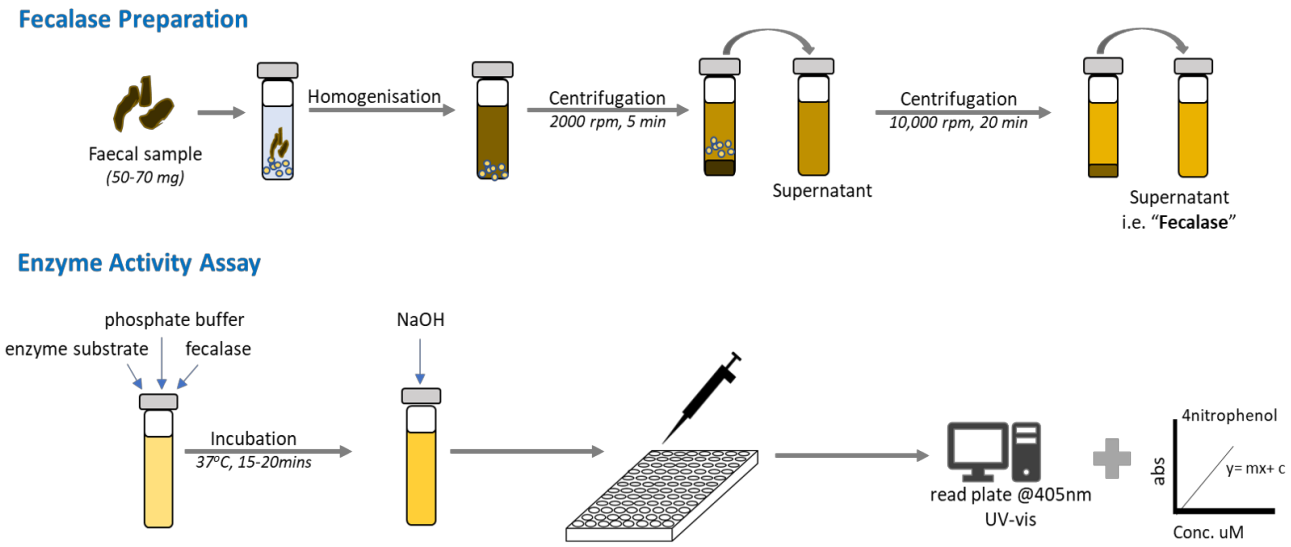

Figure 1. Method overview of fecalase preparation and enzyme activity assay. Freshly collected or fresh-frozen fecal pellets were weighed and homogenized in phosphate buffer pH7.4. Following two centrifugation steps, the supernatant was isolated (fecalase) and used in a colorimetric-based enzymatic assay. After incubation of fecalase, buffer, and a p-nitrophenyl related enzyme substrate at 37 ºC for 15 min, or 20 min with human fecalase, the reaction was stopped with sodium hydroxide (NaOH). The absorbance of the samples in a 96 well plate was read at 405nm using a UV-vis spectrophotometer, and calculations were based on a 4-nitrophenol standard curve.

Quantification of enzymatic activity and protein

β-glucosidase and β-glucuronidase activity were detected using modified protocols (36, 68) (*Figure 1*). In brief, the reaction mixture, containing 50 µl fecalase, 100 µl potassium phosphate buffer (pH7.4, 0.01M) and 100 µl 4-nitrophenyl-β-D-glucopyranoside (1mM, in buffer; Sigma Aldrich, Cas no. 2492-87-7) for β-glucosidase, or 100 µl 4-nitrophenyl-β-D-glucuronide (1mM, in buffer; Sigma Aldrich; Cas no. 10344-94-2) for β-glucuronidase, was incubated at 37 ºC for 15 min, or 20 min for human fecalase samples due to the darker color of human fecal pellets. After incubation, 250µl sodium hydroxide (NaOH) (0.5N) was added to stop the reaction, and the absorbance was measured at 405nm (UV-vis spectrophotometer).

The Pierce BCA Protein Assay Kit (ThermoFisher; Cas no. 23225) was used, following the manufacturers' protocol, to measure the total protein concentration in the fecalase samples.

Enzyme activity was calculated after correction for controls (to account for the background fecalase absorbance), from a standard curve of 4-nitrophenol (Sigma Aldrich; Cas no. 100-02-7). The unit of activity, normalized to fecalase protein, was expressed as the amount required to catalyze the formation of 1 µmole of p-nitrophenol per min under the standard assay conditions (i.e., U/mg protein).

Fecalase-mediated deconjugation of 5-HT-GLU

To assess the functional consequences of altered enzymatic activity, fecalase-mediated metabolism of 5-HT-GLU was examined. The reaction mixture, containing 50 µl fecalase, 200 µl potassium phosphate buffer (0.01M, pH 7.4) and 50 µl 5-HT-GLU (40 µg/ml, in HPLC grade water; Toronto Research Chemicals, Cas no. S274990), was prepared in duplicates and incubated at 37 ^{o}C for 1 h as per a method described by (23). Experimental controls (fecalase and 5-HT-GLU) were run simultaneously with the analyzed samples. After an aliquot was taken at T0 and T1, the reaction was stopped with 0.5N NaOH (1:1). Before HPLC analysis with an electrochemical detector (HPLC-ECD), an equal volume of an internal standard (n-methyl 5-HT, 2 ng/20 µl in HPLC mobile phase; Sigma Aldrich, Cas no. 1975-81-1) was added to each sample and vortex-mixed. The total volume (300 µl) was then transferred to the HPLC vial, and 20 µl of the final sample was injected onto the column for analysis. The method for the HPLC-ECD quantification of the parent compound 5-HT was based on a previously published method (7).

Statistical analysis

Power analysis was performed beforehand using the Software G*Power (independent two-sided t-test, or ANOVA as appropriate) to ensure adequate sample size number to detect changes in enzymatic activity or liberation of free 5-HT. All datasets were checked for

normality (Shapiro-Wilk test) and homogeneity (Levene's test). Statistical significance, defined as $p<0.05$, was determined by t-test or one-way ANOVA as appropriate for datasets containing two or more groups, respectively. A two-way ANOVA test was used to assess the impact of sex and age. Antibiotic effects were assessed using repeated-measures (RM) ANOVA with Fishers LSD test: one-way for antibiotic-associated 5-HT-GLU deconjugation data and two-way for associated enzymatic activity readouts. All data are expressed as means + SEM. Outliers were identified using the Grubbs method (22). GraphPad Prism version 6 was the software package used for statistical analysis.

Results

Impact of host-related factors on fecalase activity

Host genetics differentially alters β-glucosidase, but not β-glucuronidase, activity

C57BL/6 and BALB-c inbred mouse strains are amongst the most widely used animal models for *in vivo* experimental studies (16). As we aimed to compare enzymatic variation in outbred and inbred strains, fecalase was also prepared from National Institutes of Health (NIH)-Swiss Webster mice, as an outbred mouse model. To establish whether immunological disturbances affected the metabolic activity of gut bacteria, we also collected and processed fecal samples from genetically modified mice, including caspase-1 KO and IL-10-deficient mice, all on a C57BL/6 background. The caspase-WT strain was included as an additional control.

While β-glucuronidase activity was relatively similar across the different strains, β-glucosidase activity was significantly higher in C57BL/6 mice than in NIH-Swiss Webster mice and caspase-KO mice ($p<0.05$) (*Figure 2A*). Notably, NIH-Swiss Webster mice were associated with the lowest activity in both enzymes tested. C57BL/6 mice were subsequently used to evaluate the influence of further intrinsic and extrinsic factors on fecal metabolic activity. A deficiency in caspase-1, known to significantly alter the abundance of Firmicutes and Bacteroidetes phyla (5), was associated with a significant reduction in β-glucosidase relative to C57BL/6 mice ($p<0.05$).

Sex and age dictate β-glucuronidase activity in mice

β-glucuronidase activity in adolescent male and female C57BL/6 mice was compared to adult aged counterparts (*Figure 2B*). Increasing age was associated with a significant reduction in β-glucuronidase activity in females ($p<0.05$). In contrast, no significant difference in enzyme activity of males in the different age groups was observed. Sex differences in enzymatic activity

were only seen in the adult age group, where male C57BL/6 mice had significantly higher β-glucuronidase activity than females ($p<0.05$). To assess the physiological and metabolic implications of this latter finding, we examined if the cleavage of the glucuronide metabolite of an endogenous compound, 5-HT-GLU, was differentially affected after incubation with fecalase generated from the male and female adult mice. The reduced (~ 5-fold decreased) level of 5-HT associated with female adult fecalase relative to male adult fecalase did not reach statistical significance (*Figure 2B*).

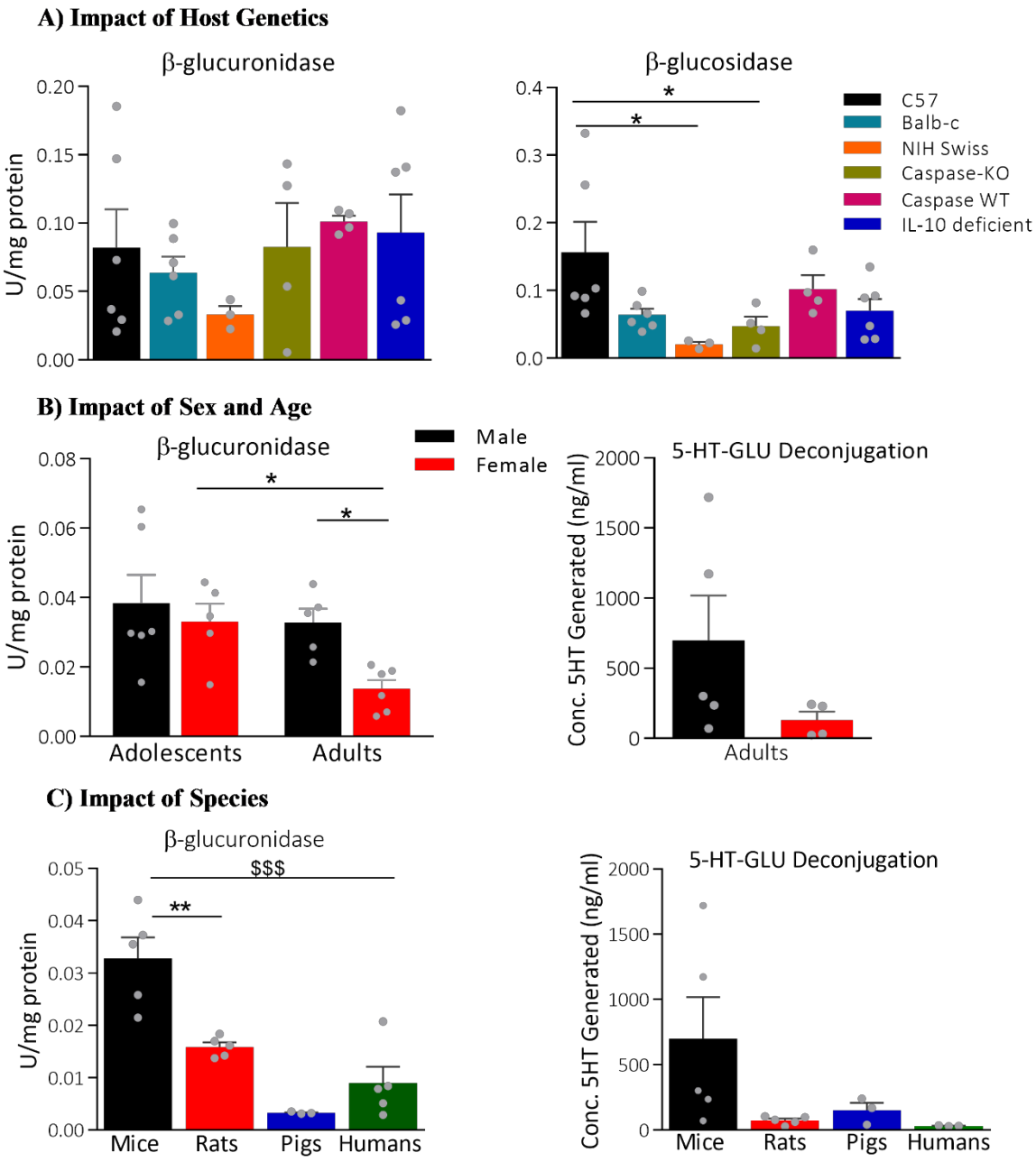

Figure 2. Host-related factors alter fecal enzymatic activity (A) Impact of host genetics. β-glucuronidase and β-glucosidase activity in male, adult (~ 10-12 wk) mice of different genetic backgrounds (n= ~ 5-6 mice/strain; KO, knockout; IL, interleukin). **B)** Impact of sex and age. β-glucuronidase activity readout in adolescent and adult-aged male and female C57BL/6 mice and bar chart representation of the amount of serotonin (5-HT) generated after the incubation of male and female adult fecalase with serotonin β-D-glucuronide (5-HT-GLU), n=5-6/group. **(C)** Impact of species on β-glucuronidase activity, as assessed in male C57BL/6 mice and Sprague Dawley rats (both ~ 8-10 wk), male landrace pigs (~ 10-12 wk), and humans (male/female samples of age-range 43 to 76 yr), and bar chart representation of the amount of 5-HT generated after incubation of fecalase from the different species with 5-HT-GLU, n=3-6 subjects/species. All data represented as mean + SEM and datasets analyzed using multiple t-tests (species and host-genetics data; the enzymatic activity of C57BL/6 mice acted as baseline

comparator for other data subjects) or two-way ANOVA (sex and age dataset; coupled with Fisher least significant difference post-hoc analysis). *, $p<0.05$; **, $p<0.01$; $$$, $p<0.001$.

Species of frequently used experimental models significantly affects β-glucuronidase activity

β-glucuronidase activity was detectable in the fecalase prepared from male Sprague Dawley rats, landrace pigs, C57BL/6 mice, and human fecal samples *(Figure 2C)*. The different species showed variable levels of enzymatic activity. The activity of β-glucuronidase was markedly decreased in the rat ($p<0.01$), pig ($p<0.001$), and human fecalase ($p<0.001$) relative to murine fecalase. Notably, no significant difference was observed in rat or pig enzymatic activity compared to the human. 5-HT-GLU deconjugation varied across species but did not reach statistical significance.

Impact of the microbiota and microbiota-targeted interventions on fecalase activity

GF status diminishes fecal enzymatic activity

The metabolic activity of feces collected from GF mice was examined to confirm that fecalase activity is microbial-derived. No detectable enzymatic activity in GF C57BL/6 mice was found in comparison to the conventional counterparts (*Figure 3A*). Moreover, de-glucuronidation of 5-HT-GLU was markedly decreased in GF mice (*Figure 3B*).

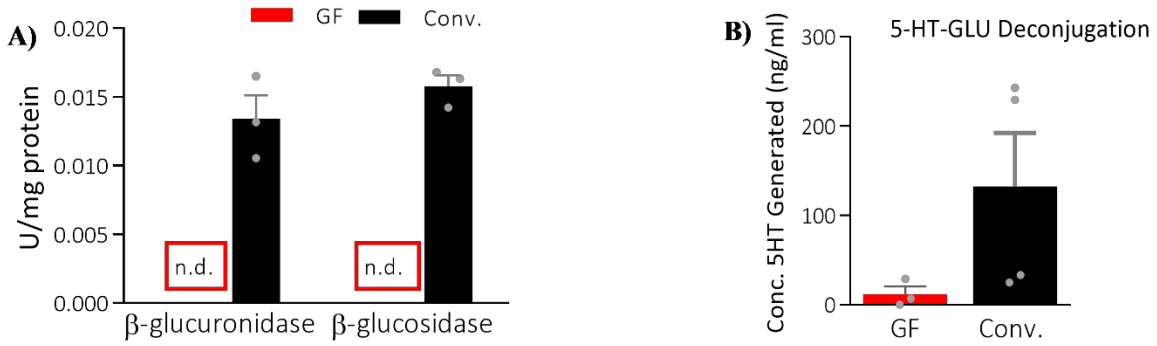

Figure 3. Fecal enzymatic activity in germ-free (GF) mice (A) β-glucuronidase and β-glucosidase activity in female GF mice compared to age- and diet-matched conventional (conv.) mice (n=3 mice; n.d., not detected). **(B)** Bar chart representation of the amount of serotonin (5-HT) generated after incubation of 5-HT-GLU with fecalase from GF and conventional mice fecalase (n=3-4 mice). Data analyzed using t-test, with significance level set at $p<0.05$.

Antibiotics significantly reduce fecal enzymatic activity during administration and after cessation of treatment

In microbiome-based studies, cocktails of antibiotics have been administered to conventional mice to induce widespread depletion of gut bacteria and act as an alternative to the GF model, termed the Pseudo-GF state (40). Our results revealed that both antibiotic cocktails decreased β-glucuronidase and β-glucosidase activity in adolescent-treated (*Figure S1; https://doi.org/10.6084/m9.figshare.11687097*) and adult-treated mice *(Figure 4B, 4D)*. Of note, during the experimental time-period of both studies, interday variability in fecal enzymatic activity was evident in the control animals.

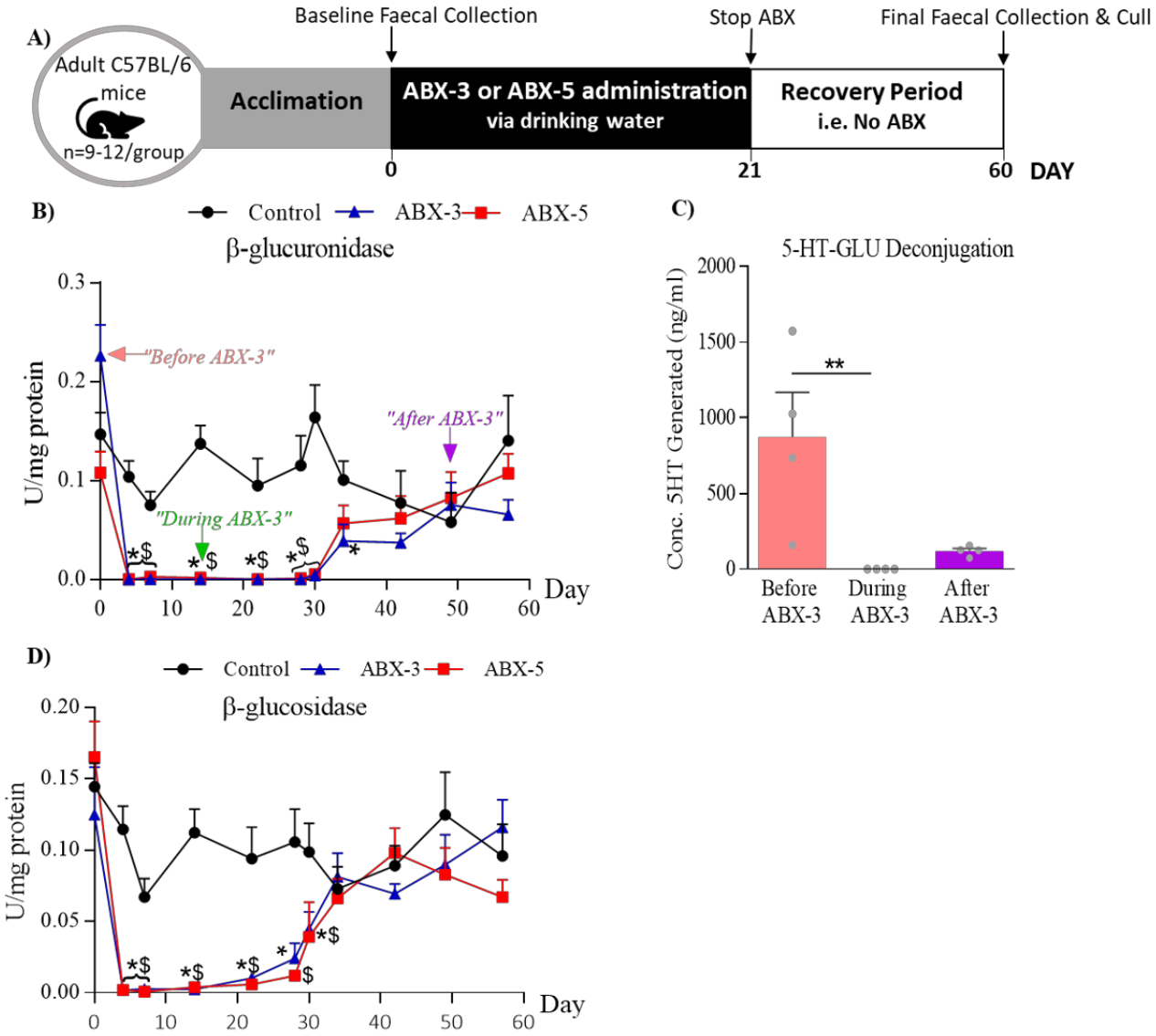

Figure 4. Fecal enzymatic activity in antibiotic-induced microbiota-depleted mice (A) Experimental timeline of the adult (72 days at baseline) antibiotic-cocktail study. **(B)** β-glucuronidase activity in antibiotic-treated mice compared to control mice (n= 9-12 mice; arrows illustrate time-points where fecalase generated from ABX3 mice were used in subsequent incubation experiment). **(C)** Bar chart representation of **t**he amount of 5-HT generated after incubation of 5-HT-GLU with fecalase from a subset of ABX-3 mice before, during, and after antibiotic treatment (n=4/timepoint). Data analyzed by repeated measures one-way ANOVA with Fishers LSD (**p<0.01). **(D)** β-glucosidase activity in antibiotic-treated mice compared to control mice (n= 9-12 mice). **(B, D)** Data represented as mean + SEM and analyzed using repeated measures two-way ANOVA with Fishers LSD test. * or $ represents p<0.05 comparison between control and ABX3 or ABX5, respectively. ABX-3: cocktail of three antibiotics [comprised of ampicillin (1mg/ml), vancomycin (5mg/ml) and neomycin (10mg/ml)]; ABX-5: cocktail of five antibiotics [comprised of ampicillin (1mg/ml), vancomycin (5mg/ml), ciprofloxacin hydrochloride (0.2mg/ml), imipenem (0.25mg/ml) and metronidazole (1mg/ml)].

Within four days of starting the antibiotic-cocktail, there was no detectable activity of either enzyme. β-glucuronidase remained significantly depleted at several different timepoints throughout antibiotic treatment in both adolescent (*Figure S1A; https://doi.org/10.6084/m9.figshare.11687097)* and adult (*Figure 4B)* mice. While β-glucosidase activity similarly remained reduced during antibiotic treatment relative to the non-antibiotic treated controls, the activity of this enzyme began to recover quicker after cessation of antibiotic than observed in β-glucuronidase, in both the adolescent (*Figure S1B; https://doi.org/10.6084/m9.figshare.11687097)* and adult mice (*Figure 4D*). Notably, the activity of these enzymes was maintained at significantly reduced levels in the antibiotic-treated groups even after administration ceased. Only on experimental day 34 or 42 (i.e., 13- or 21-days post-antibiotic treatment), did the activity of β-glucosidase and β-glucuronidase, respectively, return to similar levels of non-antibiotic mice.

The two antibiotic cocktails induced similar changes in enzymatic activity in both the adolescent and adult studies (i.e., both depleted enzymatic activity to the same extent), and in the adult-treated groups, the enzymatic activity recovered in a similar pattern after both antibiotic cocktails. Based on this similarity, the functional implication of antibiotic-depleted enzymatic activity was solely investigated in one group of antibiotic-treated mice (i.e., ABX-3). Lower 5-HT levels were observed following the incubation of 5-HT-GLU with fecalase from antibiotic (ABX-3)-treated mice as compared to baseline fecalase from the same mice ($p<0.01$) (*Figure 4C*).

Prebiotic supplementation does not alter fecal enzymatic activity

Having illustrated fecalase is extensively influenced by depletion of the gut microbiota, we subsequently explored whether the β-glucuronidase or β-glucosidase activity could be manipulated by modulating the gut microbiota with a prebiotic dietary intervention. A recent

study by our research group elucidated that chronic treatment with the prebiotic, 10% FOS-inulin, altered the composition of the gut microbiota (4). Following our findings illustrating β-glucuronidase did not differ based on the age of male mice, we collected fecal pellets only from the middle-aged male mice in this study.

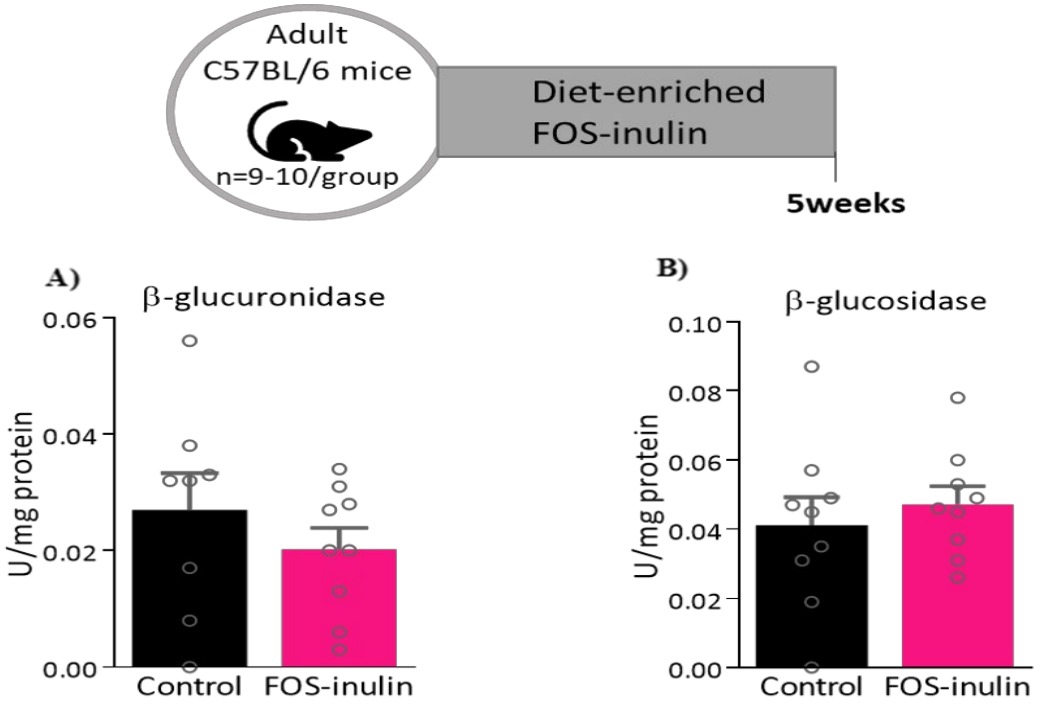

Figure 5. Experimental timeline and the impact of a prebiotic mix on fecal enzymatic activity. Middle-aged (10 mo) C57BL/6 mice received diet-enriched with 10% fructo-oligosaccharide (FOS)-inulin or a control diet for 5 wk (n=9-10). **(A)** β-glucuronidase and **(B)** β-glucosidase activity in the fecalase of FOS-inulin-treated mice versus control mice. Data represented as mean + SEM and analyzed using t-test.

Although the prebiotic mix reduced β-glucuronidase, the effect was not found to be statistically significant (*Figure 5A*). Similarly, FOS-inulin supplementation did not alter β-glucosidase activity *(Figure 5B*).

Discussion

Our results demonstrate the enzymatic activity of β-glucuronidase, and β-glucosidase depends on the species and the specific strain of the experimental model under investigation. β-glucuronidase also varies based on sex, and age in a sex-dependent manner. Bacterial-derived enzymatic activity is dependent on the microbiota and can be modulated by antibiotics, while prebiotics, with demonstrated effects on the microbiota composition (4), had a minimal impact.

Gut microbial β-glucuronidase enzymes dictate drug efficacy and toxicity, intestinal carcinogenesis, and mammalian-microbial symbiosis (10). Fecalase provides a valuable tool to identify differences in the activity of this and other microbial-derived enzymes due to a range of intrinsic and extrinsic factors. The inter-individual genetic differences in the microbiota far exceed that of our human genome (3, 47), yet knowledge of the specific influence of host genetics on fecal enzymatic activity is limited. Here, we demonstrate that mice of different genetic backgrounds (for example, C57 and NIH-Swiss Webster) have altered β-glucuronidase and β-glucosidase activity, highlighting that host genetic variability may also influence microbial metabolism. As genetic polymorphisms in host drug-metabolizing enzymes can influence the clinical outcome of approximately 20 to 25% of all drug therapies (25, 62), there is considerable scope for genetic-associated differences in fecal enzymatic activity to exert similarly extensive effects on therapeutics or the function of endogenous compounds. Indeed, previous reports have suggested that the genetic background of mice may exert a more considerable influence on the composition of the gut microbiota than environmental-associated factors (34). Significant inter-strain differences in therapeutic response to the selective serotonin reuptake inhibitors (SSRIs) fluoxetine, citalopram, and paroxetine were evident in DBA/2J and C57BL/6J (27), albeit these differences were pharmacodynamic-related. SSRIs act to accumulate 5-HT by strongly and selectively binding with 5-HT transporters (27). While

the underlying mechanism of these strain differences remains unclear, genetic-induced changes in the activity of microbial enzymes merit investigation both on the drugs *per se* and on the availability of free 5-HT in the gut.

Sex and age are factors known to introduce variability in drug metabolism (66) and the composition of the gut microbiota (4, 42, 51), we hence also determined whether these factors conferred variability in β-glucuronidase. Given their effects can be obscured by host genetics, a single strain of mice, C57BL/6, which exhibited the highest enzymatic activity, was used. Our data illustrates age-dependent effects on the activity of β-glucuronidase in female mice while differences in enzyme activity based on sex were only apparent in adult aged mice. Although host-derived serum β-glucuronidase activity has also been shown to be higher in men than in women (35), a recent protein structure-guided metagenomic study found no difference in the gene composition of microbial-derived β-glucuronidase in mice based on sex. The authors did identify differences within this comprehensive mouse gut metagenome, generated from several mouse strains, providers, housing conditions, and diets, based on mouse strain and high-fat diet (10). Enzyme activity does not always, however, correlate with gene composition or abundance (48). While sexual dimorphism is quite apparent in rodent models of drug metabolism, sex differences in hepatic drug metabolism in people are rarely encountered and generally result in relatively small effects (9, 62).

Our study shows that the fecalase assay can be used to detect and investigate the metabolic activity of the gut microbiota found in mice, rats, pigs, and humans. Previously, substantial differences in β-glucuronidase proteins between mice and humans were found at the amino acid sequence level (approximately 10% of β-glucuronidase proteins identified in the human microbiome project were identical to those recently sequenced in mouse), but a shared overall functional capacity has been suggested (10). Even though mice had the highest β-glucuronidase activity, our pig and rat data reinforce the translatability of these species to humans. This

concept is supported by findings from Yoo *et al.* who found comparable lovastatin metabolism profiles evident from rat and human fecalase, further indicating a similarity between rat and human fecalase and bacterial-derived metabolic activity (68). The age of transition between human life-stages (infancy, adolescence, adulthood, middle-age, and old-age) remains a controversial topic (13), which exacerbates the difficulty in the appropriate age-matching of animal and human samples in pre-clinical research. The human fecalase prepared herein derived from fecal samples of male and/or female study participants with a broad age-range, therefore caution is warranted in the interpretation and extrapolation of the rodent data into equivalently segregated age groups for human studies. Hence, although the purpose of this experiment was not to precisely age and sex match the samples, the results herein illustrate the important utility of fecalase as a screening platform for microbial metabolism across species.

On the other hand, Rowland *et al*. also investigated the variability in β-glucuronidase and β-glucosidase in humans and a range of animals, including mice, rats, hamsters, guinea-pigs, and marmosets. Contrary to our results, rats had significantly higher β-glucuronidase than mice, with the latter more similar to that of humans (49). The use of cecalase, the cecal-derived equivalent to fecalase, in Rowland *et al*., could account for such differences and may represent the segmental variations in the microbiota (14), and thus perhaps also microbial enzymatic activity (56), in the gut. Moreover, Rowland and colleagues prepared their enzymatic fractions under anaerobic conditions, perhaps more closely mimicking the luminal environment, although preparation under anaerobic conditions had no influence in other studies (11, 31).

Unlike the fixed host genome, the gut microbiome is readily modifiable (54, 61); thus, microbiota-targeted interventions, including prebiotics and antibiotics, may have the capacity to alter the metabolic activity of the gut microbiota. As the gut microbiome displays diurnal variations in composition (58), change in fecal collection times could account for the degree of

day-to-day variability in the activity levels of both β-glucuronidase and β-glucosidase in the non-antibiotic control animals, though our samples were collected within a two-hour window. Nevertheless, a clear difference in the antibiotic-treated and control animal enzymatic activity was observed in this study and suggested that antibiotic cocktails can be used to deplete the activity of microbial-derived metabolic enzymes to GF levels.

Recovery of β-glucosidase was evident earlier than β-glucuronidation in both the adolescent and adult studies suggesting the bacterial strains expressing or encoding β-glucosidase may be more resilient. Whole genome shotgun sequencing could shed further light on whether such bacterial strains recover quicker than β-glucuronidase associated bacterial strains. Of note, however, these enzymes also differ in their overall function. β-glucuronidase cleaves glucuronic acid from both endo- and xeno-biotics thereby exerting a predominant role of (de)toxification (12). β-glucosidase substrates are, on the other hand, mainly plant glycosides (41), and its role is linked to a plethora of biological pathways including degradation of structural and storage polysaccharides, cellular signaling, oncogenesis, host-pathogen interactions (2). Although there remains an unmet need to identify the distribution of these enzyme activities among the bacterial members of the gut microbiota, it has been proposed that β-glucosidases are more widespread in the colonic bacteria than β-glucuronidases (41). One may speculate, therefore, that these metabolic enzymes may be subject to different regulatory processes based on evolutionary principles. Even so, activity levels of both enzymes remained significantly reduced in antibiotic-treated mice for nearly 2 wk after stopping the administration of both antibiotic cocktails. Thus, we provide evidence of a suitable time window to study the impact of β-glucuronidases. This time-window could prove particularly useful for pharmacokinetic-based studies exploring microbiota-mediated drug metabolism, wherein the associated lag period, during which time most antibiotics are likely to be cleared

from the systemic circulation, could be exploited to limit any confounding drug-antibiotic interactions.

Our data suggest that not all microbiota-targeted interventions significantly affect the metabolic fraction of feces. In our study, a prebiotic mix, known to induce changes in the composition and function of the gut microbiota (4), did not alter the metabolic activity of β-glucuronidase or β-glucosidase. Both the dose and duration of treatment may be important determinants of enzymatic activity as previous studies demonstrated that inulin-containing diets significantly increased β-glucosidase activity and reduced β-glucuronidase activity, albeit species differences could also account for such variances in prebiotic response between the studies (50). Moreover, the combination of the prebiotic with the probiotic strain *Bifidobacterium longum* exerted the most substantial effect on both enzymes tested, the overall impact of these interventions (inulin-only and inulin in combination with the probiotic strain) on the composition of the gut microbiota was, however, not investigated (50). Others have highlighted that the impact of probiotics on fecal enzymatic activity is likely to be strain specific. For example, a previous study showed that a probiotic, in the form of a fermented oatmeal drink containing *Lactobacillus plantarum* 299v, did not alter β-glucosidase and β-glucuronidase activity (20). In contrast, *L. reuteri* and *L. rhamnosus* in mice significantly altered the fecal activity of sulfatase, arylsulfate sulfotransferase, and β-glucuronidase with implications for the pharmacokinetics of acetaminophen (32).

Microbiota-dependent effects on gut 5-HT significantly impact host physiology, GI motility, and platelet function (67). Accumulating evidence in GF rodents, animals devoid of a microbiota, now points to the glucuronide-conjugate of 5-HT as an endogenous substrate of bacterial-derived β-glucuronidase (23). Hata and colleagues previously showed the microbiota dictates the luminal availability of free 5-HT as there was a 5-fold reduction in the

concentration of free-5HT observed in the gut lumen of GF mice relative to ex-GF mice (23). Like our *ex vivo* data, the authors observed high variation in the luminal and tissue levels of 5-HT in the colon of ex-GF mice relative to GF mice. In this study, we assessed the conversion of 5-HT-GLU to 5-HT by β-glucuronidase to gain insight into the physiological relevant functional activity of our fecal fractions. Our findings highlight that intrinsic or extrinsic factors, which modify β-glucuronidase activity, may alter the luminal availability of 5-HT in the gut which may, in turn, have a direct consequence on the physiological processes or GI functions on which serotonin exerts its' effect including peristaltic reflexes and contractile frequency (26, 29) (*Figure 6*).

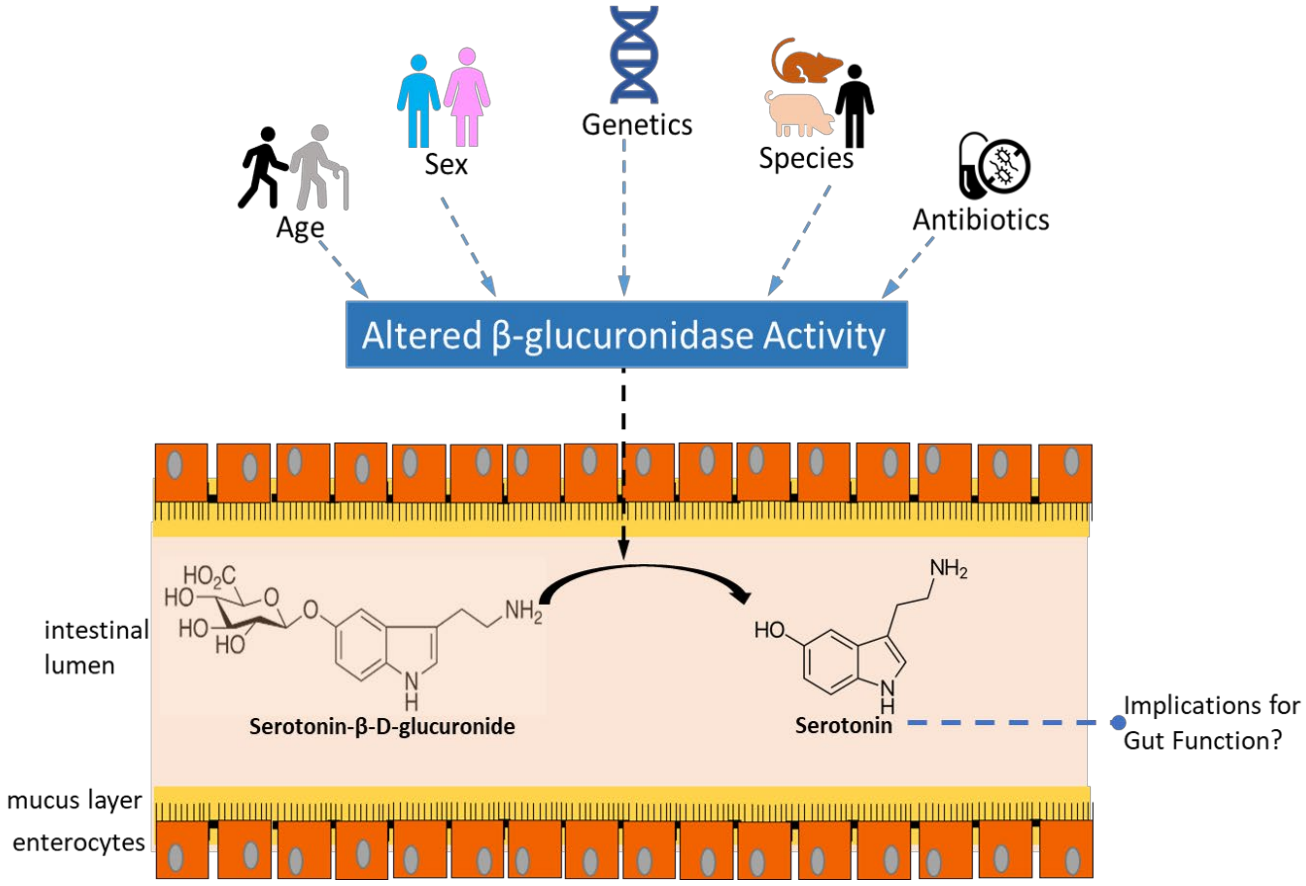

Figure 6. Summary of factors influencing β-glucuronidase activity. Our results suggest age, sex, host genetics, species, and antibiotic-induced microbiota depletion can modify bacterial-derived enzymatic activity. Altered β-glucuronidase activity may alter the amount of free serotonin in the gut lumen with knock-on effects on gut motility and function.

The variability observed in the readouts of enzymatic activity (41), and the liberation of 5-HT (23) are in line with the values in the published literature. This experimental approach could be extended to investigate the impact of intrinsic and extrinsic factors on other endogenous glucuronides in the gut including bilirubin (60), catecholamines (1) and sex hormones (64). For example, recent work by Ervin and colleagues investigated β-glucuronidase-mediated metabolism of estrogens. While the author's hypothesis that a β-glucuronidase-rich gut microbiota, shown to increase the reabsorption of free estrogens, may contribute to the pathology of breast cancer, was unproven, it was proposed that endogenous estrogens are transformed and repurposed for use in other areas of the body, like distal mucosal or receptor sites (15). Taylor and colleagues recently illustrated vancomycin-induced depletion of β-glucuronidase attenuated the GI toxicity of mycophenolate mofetil (57). Others, in contrast, have focused on the investigation and development of selective inhibitors of microbial β-glucuronidase as a more targeted pharmacological strategy to circumvent the GI toxicity of drugs liable to β-glucuronidase-mediated biotransformation for example, irinotecan, an anticancer-drug (37), and diclofenac, a non-steroidal anti-inflammatory drug (38, 52).

It is important to point out that microbial enzymatic activity was estimated *ex vivo* rather than *in vivo*. The *in vivo* metabolism of a substrate by the gut microbiome is dependent on several other factors, which are not accounted for in our fecalase preparations, including the amount and rate of entry of the substrate into the gut, transit time, pH, and redox potential (49). This model may, hence, be overly simplistic and may not fully recapitulate the unique features of the gut ecosystem (8). This research provides the impetus for future *in vivo* proof-of-concepts studies to further confirm such a hypothesis and its physiological implications on the host.

The transplantation of fecal material (i.e., FMT) with varying levels of β-glucuronidase into GF mice and subsequent analysis of luminal levels of serotonin within the different regions of

the GI tract could provide additional evidence of altered levels of enzymatic activity precipitating changes to the systemic availability of this endobiotic. However, there are complex potential confounding factors to consider in relation to the altered 5-HT system in GF mice (67), and it would be challenging to tease apart the respective contribution of FMT in GF animals on the host and microbial contributions to free luminal 5-HT. Increasing the concentration of free 5-HT liberated is functionally relevant as previous studies have illustrated that luminal administration of 5-HT significantly accelerates both colonic transit and motility (17, 59). Increases in free luminal 5-HT have also been shown to activate mucosal 5-HT4 receptors leading to propulsive motor activity (53), and to impact on colonic anion secretion (28). The impact of microbial diversity on enzymatic activity could be further explored beyond the relatively mild, but more clinically relevant, FOS-inulin intervention employed herein. Furthermore, colonization with a defined murine microbial community, such as the altered Schaedler flora (ASF) (65), may lend additional support to the microbiota-driven effects on enzymatic activity. Such an alternative approach has, however, been linked to the normalization of phenotype in GF animals (65).

We propose that fecalase is a valuable screening assay to acquire insight into bacterial-mediated metabolism of xeno- and endo-biotics, the impact of microbiota-targeted interventions on the metabolic activity of the gut microbiome, and factors that influence free 5-HT in the gut. We illustrated that antibiotic-induced depletion of the metabolic activity of the gut microbiota could be a useful and more accessible tool, as an alternative to the GF model in gut microbiota-related metabolic studies and more specifically pharmacokinetic studies. Our findings demonstrate that fecalase is sensitive to factors, such as age, which underpin variability in physiological function or drug response. Further studies are required to link the biochemistry of microbiota enzymes with mammalian physiology (10) and to extrapolate our 5-HT findings to glucuronide-conjugates of other xeno- and endo-biotics.

List of Abbreviations:

ABX, antibiotic; ASF, altered Schaedler flora; Conv, conventional; ECD, electrochemical detection; GF, germ-free; IL, interleukin; FMT, fecal material transplantation; FOS; fructo-oligosaccharide; GI, gastrointestinal; I.S., internal standard; KO, knockout; NaOH, sodium hydroxide; SEM, standard error of the mean; SSRIs; selective serotonin reuptake inhibitors; RM, repeated measures; 5-HT, Serotonin (5-hydroxytryptamine); 5-HT-GLU, Serotonin β-D-Glucuronide; ABX-3, cocktail of three antibiotics [comprised of ampicillin (1mg/ml), vancomycin (5mg/ml) and neomycin (10mg/ml)]; ABX-5, cocktail of five antibiotics [comprised of ampicillin (1mg/ml), vancomycin (5mg/ml), ciprofloxacin hydrochloride (0.2mg/ml), imipenem (0.25mg/ml) and metronidazole (1mg/ml)].

Conflict of interest:

J. F. Cryan & T.G. Dinan have research funding from Dupont Nutrition Biosciences APS, Cremo SA, Alkermes Inc, 4D Pharma PLC, Mead Johnson Nutrition, Nutricia Danone, Suntory Wellness. JFC, TGD & GC have spoken at meetings sponsored by food and pharmaceutical companies. All other authors report no financial interests or potential conflicts of interest.

Author Contributions:

J.W., L.O.-R., G.L., M.B., T.G.D., J.F.C., B.T.G., N.P.H., and G.C. conceived and designed research; J.W., L.O.-R., and G.L. performed experiments; J.W., L.O.-R., and G.C. analyzed data; J.W., B.T.G., N.P.H., and G.C. interpreted results of experiments; J.W. prepared figures; J.W. drafted manuscript; J.W., M.B., T.G.D., B.T.G., N.P.H., and G.C. approved final version of manuscript; G.L., M.B., T.G.D., J.F.C., B.T.G., N.P.H., and G.C. edited and revised manuscript.

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